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Award Number: W81XWH-14-1-0197

TITLE: FLT-PET/CT as a Biomarker of Therapeutic Response in Pemetrexed Therapy for Non-Small Cell Lung Cancer

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14. ABSTRACT: Pemetrexed(PEM), a standard therapy for non-squamous non-small cell lung cancer (NSCLC), inhibits the de novo thymidine pathway, resulting in a transient burst of metabolism through the salvage pathway, an effect detected as a "flare" of activity by 18F-thymidine (FLT)-PET. FLT is a reliable biomarker of proliferation, and post-therapeutic changes in tumor FLT avidity predicts therapeutic response in a range of malignancies. However, FLT as a measure of therapeutic response in NSCLC is not well studied. Our overarching hypothesis is that FLT-PET will allow early assessment of therapeutic response in NSCLC with the following specific aims: Specific Aim 1: To determine the optimal timing to measure FLT "flare", an imaging biomarker of successful PEM-induced TS inhibition in a NSCLC preclinical model. Specific Aim 2: To conduct a proof-of-concept clinical study of FLT flare and FLT-measured changes in tumor proliferation at 2 weeks of therapy as predictors of NSCLC response to PEM. During the research period, we have determined that the optimal time to observe FLT flare in vivo is at 2 hrs following therapy start. This data has been translated to an open clinical trial of FLT-PET as a biomarker of therapy response in NSCLC proposed in Specific Aim #2.

15. SUBJECT TERMS
FLT-PET, biomarker, therapy response, Non-small cell lung cancer,

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. **INTRODUCTION:**

Pemetrexed (PEM), a standard therapy for non-squamous non-small cell lung cancer (NSCLC), inhibits the *de novo* thymidine pathway, resulting in a transient burst of metabolism through the salvage pathway, an effect detected as a "flare" of activity by 18F-thymidine (FLT)-PET **(Fig. 1)**. FLT is a reliable biomarker of proliferation, and post-therapeutic changes in tumor FLT avidity predict therapeutic response within a few weeks from the start of therapy in a range of malignancies. However, FLT as a measure of therapeutic response in lung cancer is not well studied. Hypotheses/Objectives: Our overarching hypothesis is that FLT-PET will allow early assessment of therapeutic response in NSCLC. Our primary objective is to assess whether "FLT flare", a transient (<24 hrs) burst of DNA salvage pathway activity induced by TS inhibition, is predictive of PEM therapeutic success. Our secondary objective is to determine whether post-therapeutic changes in tumor proliferation, assessed by FLT-PET at baseline and at 2 weeks of PEM therapy, correlate with tumor response.

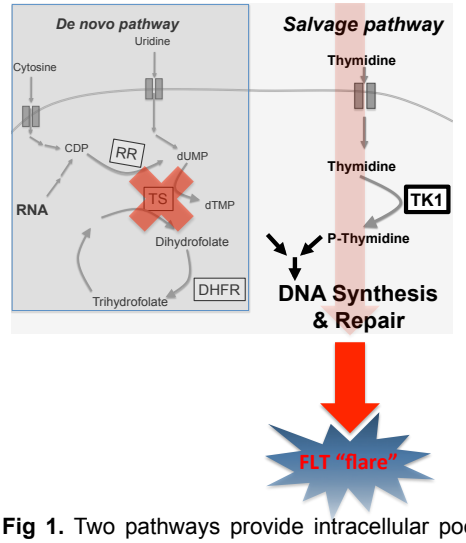

Fig 1. Two pathways provide intracellular pools of thymidine. Inhibition of the *de novo* synthetic pathway results in increased flux through the DNA salvage pathway, visible as a "flare" in FLT avidity. FLT is metabolized and trapped in the cell by the DNA salvage pathway

Specific Aim 1: To determine the optimal timing to measure FLT "flare" as an imaging biomarker of successful PEM-induced TS inhibition in a NSCLC preclinical model.

SubAim 1.1 To validate xenograft chemotherapeutic regimen of PEM-induced FLT flare in a NSCLC xenograft mouse model.

SubAim 1.2 To determine the kinetics of FLT "flare" in xenograft model of NSCLC.

Specific Aim 2: To conduct a proof-of-concept clinical study of FLT flare and FLT-measured changes in tumor proliferation at 2 weeks of therapy as predictors of NSCLC response to PEM.

2. **KEYWORDS:** FLT-PET, biomarker, non-small cell lung cancer, clinical trial, mouse model, therapy response, imaging

3. **ACCOMPLISHMENTS:**

Major Task 1: Training and educational development in lung cancer research	**Projected Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Meet biweekly with Drs. Schnall (primary mentor) and Albelda (co-mentor) and monthly with co-mentors Drs. Mankoff and Langer	1-24	100%		I am continuing to meet with my mentoring team as scheduled.
Subtask 2: Attend weekly meetings and present research at the Penn	1-24	100%		I am continuing to

Molecular Imaging Research Group				attend these meetings and to present research.
Subtask 3: Attend monthly meetings and present research at the Tobacco and Environmental Carcinogenesis Clinical Trials Working Group	1-24	0%	n/a	This working group no longer has regular meetings
Subtask 4: Attend relevant seminars and meetings of the Abramson Cancer Center Lung Center of Excellence	1-24	100%		These seminars and meetings are ongoing, and I am continuing to attend.
Subtask 5: Attend annual ACRIN-ECOG and meetings for the Young Investigator Initiative for Conduct of ACRIN Ancillary Research	1-24	100%		I am continuing to attend these meetings.
Subtask 6: Attend annual World Congress of Molecular Imaging and one Keystone Symposium on cancer biology	1-24	0%	1-12	Anticipated for 2016
Subtask 7: Complete formal coursework at UPENN	1-24	0%	1-12	Anticipated for 2016
Milestone(s) currently Achieved: • *Integration into Thoracic Core Committee of the ACRIN-ECOG organization* • *Mature clinical research skills through formal meetings, data presentation and networking.*				*In the past year I have attended the fall and spring working group meetings for ECOG-ACRIN resulting in new collaborations with Fox Chase Cancer Center. In spring 2015 I was asked to give a talk to the Thoracic Core*

				Committee of ECOG-ACRIN regarding FLT-PET as an imaging correlate in NSCLC.

Research-Specific Tasks:

Specific Aim 1: To determine the optimal timing to measure FLT "flare" as an imaging biomarker of successful PEM-induced TS inhibition in a NSCLC preclinical model.		**100%**		
Major Task 1: To validate xenograft chemotherapeutic regimen of PEM-induced FLT flare in a NSCLC xenograft mouse model.	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Obtain USAMRMC Office of Research Protections (ORP) approval for IACUC protocol(institutional approval anticipated in advance of the start of the grant award period).	1-3	100%		
Subtask 2: NSCLC xenograft trial of PEM + cisplatin to confirm efficacy of therapeutic regimen. Cell line used: H460 [Gift of Dr. Albelda] Mice: 6 wk old mice *nu/nu* (Strain#088, Charles River Labs); (Total mice: 8 mice/group x 2 groups = 16 mice)	4-5	100%		
Subtask 3: Histologic analysis of excised tumors (CHOP Pathology Core)	5	100%	1-3	
Subtask 4: Statistical Analysis of data	5	100%	1-3	
Major Task 2: Characterization of kinetics of FLT "flare" in xenograft model	**Months**	**Percentage of Task Currently Completed**	**Anticipated Time to Completion (months)**	**Comments**

		(%)		
Subtask 2: Examine FLT "flare" kinetics in PEM-sensitive NSCLC xenografts. This will be done for 3 PEM-sensitive cell lines. Cell lines used: H1299, H460, H1264 [Gift of Dr. Albelda] Mice: 6 wk old mice *nu/nu* (Strain#088, Charles River Labs); (Total mice: 3 cell lines x 8 mice/group x 2 groups x 5 time points = 240 mice) FLT/PET: FLT (250 μCi/FLT-PET scan). Resources available through the UPENN Dept. of Radiology Cyclotron and Small Animal Imaging Facility (SAIF).	6-9	100%	1-3	
Subtask 2: Histologic analysis of excised tumors (CHOP Pathology Core)	9	100%	1-3	
Subtask 2: Statistical Analysis of data	9	100%	1-3	
Subtask 3: Submission of manuscript for publication	10-13	100%	3-6	The manuscript in currently under review.
Subtask 4: Present data at a national meeting	13-24	90%	6-12	The data will be presented as an abstract at the AACR annual meeting 2016
Milestone(s) Achieved: • *Through in vitro and completed in vivo data, we have determined that the optimal time to observe FLT flare in vivo is at 2hrs following therapy start.*				The pre-clinical aim is nearly complete and we have successfully determined the optimal timing to observe the FLT flare effect in a mouse model

				of NSCLC
Specific Aim 2: Proof-of-concept clinical study of FLT therapeutic response prediction of NSCLC response to PEM-based therapy.		10%		
Major Task 2: Prepare for opening of clinical study	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Obtain USAMRMC ORP HRPO approval of IRB protocol (IRB approval anticipated in advance of award start)	1-3	100%		
Subtask 2: Finalize choice of clinical research assistant (CRA) from available pool of Dept. CRAs.	1-3	100%		
Subtask 3: Prepare for study opening (organize study binders, define clear study algorithm and review with study personnel)	3-12	100%		
Subtask 4: Determine the optimal timing to observe the FLT "flare" from preclinical obtained through Spec Aim1. If pre-clinical data is indeterminate, FLT flare will be measured at 1hr following infusion of PEM, similar to what has been suggested for 5-FU[10] and capecitabine[12].	9	100%		
Milestone(s) Achieved: • *Select and organize study personnel* • *Finalize regulatory documents and clinical trial design*		100%		
Major Task 3: Clinical study accrual	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Begin patient recruitment. CRA to consent patients in offices of the Division of Thoracic Oncology in	10-14	100%		

collaboration with the patient's oncologist. Anticipated quarterly accrual: 20				
Subtask 2: Complete study accrual and close study enrollment.	14	10%		
Subtask 3: Complete progression-free survival data	11-20	5%		
Milestone(s) Achieved: • *Begun patient accrual for clinical trial* • *Begun of follow-up data collection on progression-free survival*				To date, 2 of the total 20 anticipated patients have been enrolled into the clinical trial.
Major Task 4: Clinical study data analysis	**Months**	**Percentage of Task Currently Completed (%)**	**Anticipated Time to Completion (months)**	**Comments**
Subtask 1: Calculate CT RECIST data for 1st restaging	14-17	0%	9-11	
Subtask 2: Calculate tumor FLT avidity from FLT-PET/CT scans. Performed on available UPENN clinical software.	11-16	0%	9-11	
Subtask 3: Statistical analysis of clinical study data	20-21	0%	9-11	
Subtask 4: Submission of manuscript of proof-of-concept study data for publication	22-24	0%	11-12	
Subtask 5: Presentation of data at a national meeting	21-24	0%	11-12	
Milestone(s) Achieved: • *These tasks are anticipated to be completed over the course of the next year*				

What was accomplished under these goals?

1) Major activities: During the period of the 1st year of this grant, the majority of the preclinical aim, Specific Aim #1 was completed in a time period similar as projected by the initial Statement of Work. During this period, the clinical trial proposed in Specific Aim #2 was opened and accrual started in line with the proposed outline from the Statement of Work.

2) Specific objectives: Our overarching hypothesis is that FLT-PET will allow early assessment of therapeutic response in NSCLC. Our primary objective is to assess whether "FLT flare", a transient (<24 hrs) burst of DNA salvage pathway activity induced by TS inhibition **(Fig. 1)**, is predictive of PEM therapeutic success. Our secondary objective is to determine whether post-therapeutic changes in tumor proliferation, assessed by FLT-PET at baseline and at 2 weeks of PEM therapy, correlate with tumor response.

3) Significant results or key outcomes: During the reporting period, a preclinical model of human non-small cell lung cancer (NSCLC) was employed to study the kinetics of the pemetrexed induced FLT "flare". *In vitro* studies of 2 human NSCLC cell lines, H460 and H1299, revealed that the activity of the DNA salvage pathway, as measured by ^{3}H-thymidine labeling, peaks 2 hours **(Fig. 2)** following the exposure of cells to pemetrexed. Furthermore this increased activity of the DNA salvage pathway appears to be the result of shift in state of TK1, the rate limiting step of the DNA salvage pathway, to a more activated tetramer state **(Fig. 3)** and the translocation of ENT1 **(Fig. 4)**, the cell surface nucleoside transporter responsible for thymidine entry into the cell, from the nucleus to the cell surface. We then examined the timing of the FLT flare *in vivo* in a xenograft mouse model of NSCLC confirmed that the FLT flare peaks at 2 hrs of exposure to pemetrexed **(Fig. 5)**. These data have resulted in a manuscript that is currently under review and presentation at a national meeting within the next year.

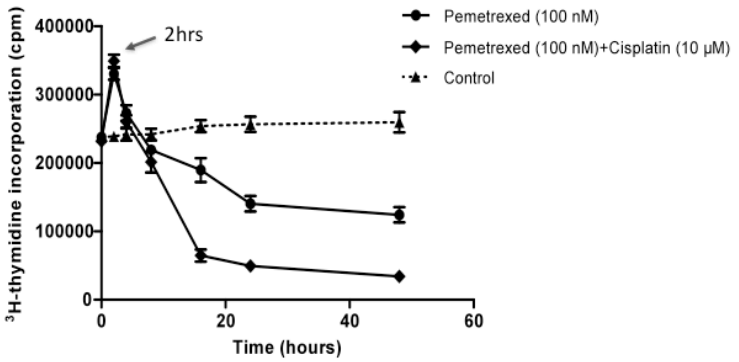

Fig 2. Pemetrexed-induced TS inhibition results in a "flare" of the DNA salvage pathway activity. ^{3}H-thymidine incorporation assay of PEM-sensitive NSCLC H460 in untreated control (culture medium only), pemetrexed (100nM) and combination therapy with pemetrexed (100nM) + cisplatin (10mM). A "flare" of DNA salvage pathway activity peaks at 2 hrs of pemetrexed therapy exposure.

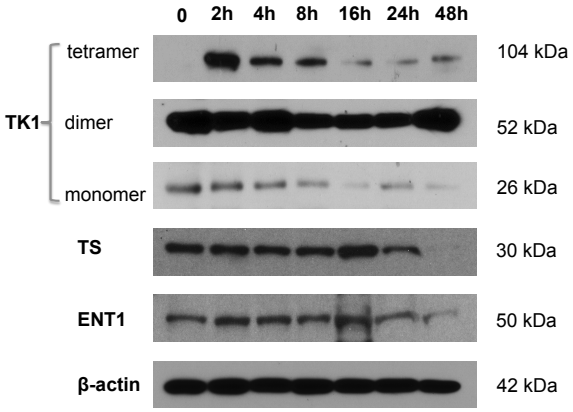

Fig 3. Induction of tetramer state of TK1 corresponds temporally with pemetrexed-induced DNA salvage pathway "flare": A time course of exposure of PEM-sensitive NSCLC cell line H460 to combination therapy with PEM/cisplatin *in vitro* reveals induction of highly activated tetramer TK1 state corresponding temporally to the FLT "flare" observed at 2hrs. ENT1 and TS protein expression levels remain unchanged in the "flare" period. All protein levels slowly decrease beyond 8 hrs due to successful cell cycle inhibition by PEM/cisplatin therapy.

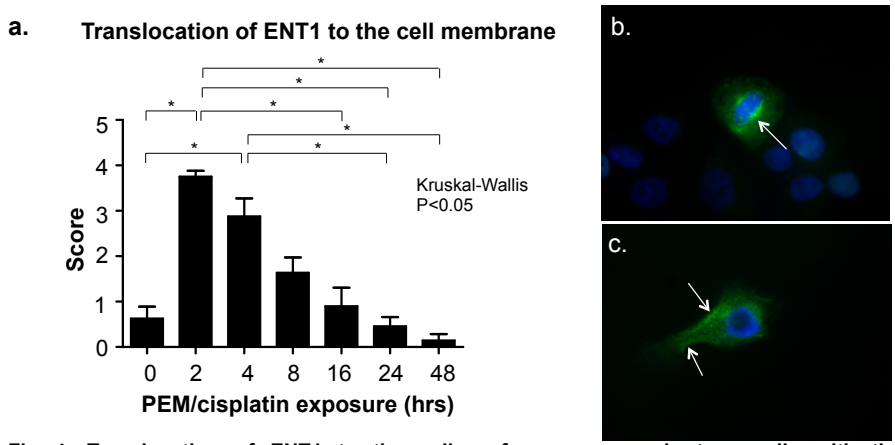

Fig 4. Translocation of ENT1 to the cell surface corresponds temporally with the pemetrexed-induced DNA pathway "flare": Immunofluorescence microscopy utilizing time course of exposure of Pemetrexed (PEM)-sensitive NSCLC cell line H460 to combination therapy with PEM(100nM)/cisplatin(μM) *in vitro* reveals translocation of equilibrative nucleoside transporter 1 (ENT1) to the cell surface from the perinuclear cytoplasm maximal at 2 hrs of exposure to PEM or PEM/cisplatin corresponding to the timing of the FLT "flare". (a.) cells were scored on a scale 0 (no ENT1 translocation) to 5 (high ENT1 translocation to the cell surface) Microscopy demonstrates NSCLC staining for ENT1 (Green stain) and nuclear membrane (blue stain) following 2 hrs of exposure to (b.) culture medium control (c.) or combination of pemetrexed and cisplatin. Arrows

In addition to the pre-clinical aim, the clinical trial of FLT-PET as a biomarker of therapy response to pemetrexed-based therapy in NSCLC has opened and recruitment begun. A total of 2 patients have been enrolled, 1 of which has completed imaging. In this clinical trial we assess the FLT "flare" as close to 2 hours following therapy start as possible and compare to tumor baseline FLT-PET signal. Our completed clinical trial patient demonstrated an FLT "flare" at 2 hrs following administration to pemetrexed **(Fig. 6)**. This demonstrates feasibility of this technique in this patient population. We will continue to enroll the remainder of the patients to this clinical trial over the course of the next year of this DOD CDA grant. In addition to assessing the FLT "flare", we will also assess the impact of pemetrexed-based therapy on tumor proliferation measured at 2 weeks following therapy start compared to baseline. Changes in tumor avidity for FLT-PET will be correlated to clinical measures of therapy response including CT RECIST, progression-free survival and overall survival. These data is anticipated to result in a manuscript and presentation at a national meeting within the next year.

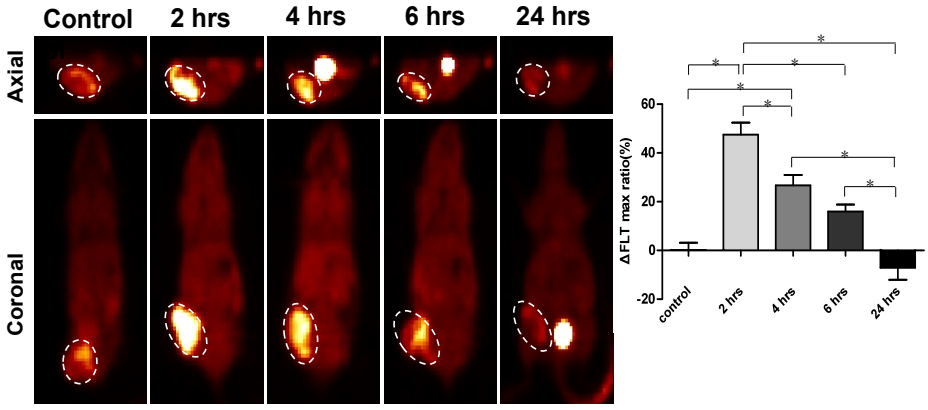

Fig 5. FLT-PET imaging of pemtrexed-induced TS inhibition demonstrates a FLT "flare" peaking at 2hrs in a preclinical model of NSCLC. Human NSCLC tumor-bearing murine xenografts were treated with a combination of pemetrexed (PEM)and cisplatin in order to model 1st line therapy. FLT-PET was performed at baseline and at multiple timepoints following therapy start. Tumor avidity for FLT was observed to peak at 2 hrs following PEM-based therapy. By 24 hrs of therapy, tumors begin to demonstrate inhibition of proliferation. (b) bladder

What opportunities for training and professional development has the project provided?

During the reporting period, Dr. Katz met routinely with her mentors, Drs. Schnall, Mankoff, Albelda and Langer as outlined in the Statement of Work. In addition, Dr. Katz has participated in the relevant meetings and seminars are outlined in the Statement of Work. These interactions have assisted in supporting the progress of both the preclinical aim and clinical trial.

In addition, Dr. Katz, under the mentorship of Drs. Schnall and Mankoff, has engaged in the ECOG-ACRIN organization integrated into the Thoracic Core Committee. Dr. Katz attended the Fall 2014 and Spring 2015 ECOG-ACRIN working group meeting. Networking in the fall 2014 meeting led to Dr. Katz giving a talk regarding

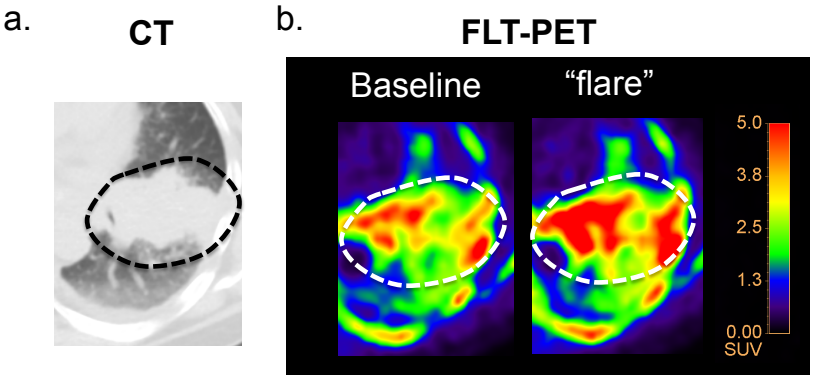

Fig. 6: FLT "flare" in response to PEM-based therapy in a human patient. These images are from a 63 y/o male with NSCLC participating in our exploratory clinical trial of FLT-PET "flare". (a.) Baseline CT reveals a 6 cm mass in the left upper lobe. (b.) Baseline FLT-PET reveals mild avidity 3 days pre-therapy. FLT-PET "flare" imaging performed after 2 hrs following administration of combination therapy with PEM and carboplatin reveals a burst in tumor avidity in keeping with the FLT "flare".

FLT-PET as an imaging correlate for therapeutic clinical trial in the Spring 2015 working group.

In addition to Dr. Katz, the Ms. Urooj Khalid, a University of Pennsylvania undergraduate student, and Mr. Ian Berger, a University of Pennsylvania Perelman School of Medicine student were engaged in the clinical trial proposed in Specific Aim #2, Under the mentorship of Dr. Katz, Ms. Khalid and Mr. Berger have had met weekly with Dr. Katz and have learned the basics of clinical trial design, have had hands on experience with patient recruitment and have been actively involved in day-to-day problem solving issues related to the clinical trial. Both of these students have submitted research abstract to the Society of Thoracic Radiology under the mentorship of Dr. Katz.

How were the results disseminated to communities of interest?
I have presented at ECOG-ACRIN, have a manuscript under review and will present an abstract to the AACR annual meeting 2016.

What do you plan to do during the next reporting period to accomplish the goals?
In the next reporting period we will accomplish the following:

- Complete accrual to the clinical trial proposed in Specific Aim#2, analyze the data, submit a manuscript and present the data at a national meeting.
- Complete the proposed training including coursework at the University of Pennsylvania.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?
The work accomplished on the pre-clinical aim succeeded in determining the optimal timing to measure the FLT-PET "flare". This information was integrated into the proposed exploratory clinical trial which is now in progress. Ultimately, if the use of the FLT-PET "flare" technique is successful in clinical trials, the information on optimal timing will be translatable to a multicenter clinical trial.

What was the impact on other disciplines?
If the use of the FLT-PET "flare" technique is successful in clinical trials, the information on optimal timing may ultimately be translatable to a clinical use in oncology and other disease entities that employ use of thymidylate synthase (TS) inhibition as a drug therapy.

What was the impact on technology transfer?
"Nothing to Report."

What was the impact on society beyond science and technology?
"Nothing to Report."

5. **CHANGES/PROBLEMS:**
"Nothing to Report"

6. **PRODUCTS:**

Publications, conference papers, and presentations

- **Journal publications.**

1 manuscript in preparation.

- **Books or other non-periodical, one-time publications.**
  "Nothing to Report."

- **Other publications, conference papers, and presentations.**
  1 abstract will be presented at the AACR annual meeting in 2016.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Sharyn Katz, MD, MTR	Xiao Chen, MD	Urooj Khalid	Ian Berger	Jenny Cai
Project Role:	Principal Investigator	Research Assistant	Student Research Assistant	Student Research Assistant	Clinical Research Assistant
Researcher Identifier (e.g. ORCID ID):					
Nearest person month worked:	9	8	2	2	2
Contribution to Project:	Dr. Katz is the project PI and leads the preclinical aim and exploratory clinical trial	Dr. Chen provides technical support in the laboratory for the preclinical aims of this project.	Ms. Khalid is an Undergraduate student at the University of Pennsylvania and assists in recruitment of potential patients to the project clinical trial.	Mr. Berger is a medical student at the University of Pennsylvania Perelman School of Medicine and assists in recruitment of potential patients to the project clinical trial.	Ms. Cai is a clinical research assistant at the Hospital of the Univ. of Pennsylvania and assists in patient recruitment, accompanying the patient on the day of the FLT scans, and maintenance of the regulatory binder.
Funding Support:	DOD LRCP CDA, American Cancer Society-	Self-funded through funds from her home university	Funded through the PI's, University of Pennsylvania University	Not funded (volunteer)	Supported through funds through the Dept. of Radiology,

	Investigator Research Grant (ACS-IRG), University of Pennsylvania University Research Fund (URF) Award	in China	Research Fund (URF) Award		Hospital of the Univ. of Pennsylvania

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
"Nothing to Report."

What other organizations were involved as partners?
"Nothing to Report."

8. **SPECIAL REPORTING REQUIREMENTS**
N/A

9. Conclusion: The work performed in the 1st year of this proposal is in keeping with the initially proposed statement of work.

Appendix 1: Submitted manuscript

Title: Early Detection of Pemetrexed-induced Inhibition of Thymidylate Synthase in Non-Small Cell Lung Cancer with FLT-PET imaging.

Authors: Xiao Chen[1,2,], Yizeng Yang[3], Ian Berger[1], Urooj Khalid[1], Akash Patel[1], Jenny Cai[1], Michael D. Farwell[1], Corey Langer[3], Charu Aggarwal[3], Steven M. Albelda[3], Sharyn I. Katz[1,4]

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Running Title: Imaging of TS inhibitor therapy response in NSCLC

Keywords: FLT, PET, pemetrexed, lung cancer, flare

Funding Source: Department of Defense, Lung Cancer Research Program, Career Development Award, LC130313

STATEMENT OF TRANSLATIONAL RELEVANCE

Most patients with lung cancer have limited survival at diagnosis. Yet current management requires months to assess therapeutic response by computed tomography. Thus, new approaches for early assessment of therapeutic response are urgently needed. In this study we delineate an imaging strategy, FLT-PET, to rapidly (within hours) assess tumor response to 1st line pemetrexed-based therapy for non-small cell lung cancer (NSCLC) or mesothelioma by determining successful inhibition of thymidylate synthase. We then successfully translate this strategy from our preclinical models into a patient with lung cancer. Overall, these data provide the foundation for a large-scale clinical trial of FLT-PET as a biomarker of response to 1st-line therapy in NSCLC and mesothelioma, which could change management of these cancers.

ABSTRACT

Purpose: Inhibition of thymidylate synthase (TS) results in a transient burst or "flare" in DNA thymidine salvage pathway activity measurable with FLT ([^{18}F]thymidine)-positron emission tomography (PET), allowing a measure of tumor response to TS inhibition on day 1 of therapy. Here we characterize this imaging strategy for implementation into 1st line pemetrexed-based therapy for non-small cell lung cancer (NSCLC) or mesothelioma.

Experimental Design: Since pemetrexed acts by inhibiting TS, we defined the kinetics of increases in thymidine salvage pathway mediated by TS inhibition following treatment with pemetrexed *in vitro*. Next, using a mouse model of NSCLC, we validated the kinetics of the pemetrexed-mediated "flare" in thymidine salvage pathway activity *in vivo* using FLT-PET imaging. Finally, we translated our findings into a proof-of-principle clinical trial of FLT-PET in a human NSCLC patient.

Results: In NSCLC cells *in vitro*, we identified a burst in thymidine salvage pathway activity, assessed by ^{3}H-thymidine assays, thymidine kinase 1 (TK1) expression, and equilibrative nucleoside transporter 1 (ENT1) mobilization to the cell membrane, that peaked two hours after pemetrexed treatment. Addition of cisplatin did not impact the amplitude or timing of this pemetrexed-induced "flare". This two-hour time-point was also optimal for FLT-PET imaging of pemetrexed-mediated TS inhibition in murine xenograft tumors. Imaging of pemetrexed-induced TS inhibition in a NSCLC patient demonstrated feasibility at the 2 hour time point.

Conclusions: FLT-PET measured efficacy of pemetrexed-induced TS inhibition is optimal at 2 hours from the start of therapy; this timing is feasible in human clinical trials.

INTRODUCTION

Successful inhibition of thymidylate synthase (TS), a critical enzyme in the *de novo* thymidine synthesis pathway, is the key target of chemotherapeutics such as 5-fluorouracil (5-FU), pemetrexed, and capecitabine. Once TS is blocked, a rapid compensatory increase in the thymidine salvage pathway occurs resulting in a rapid uptake of extracellular thymidine. This burst or "flare" in uptake can be visualized using ^{18}F-thymidine (FLT), an analogue of thymidine and a PET (positron emission tomography) radiotracer. FLT was first described as an imaging biomarker of thymidine salvage activity by Shields and Grierson *et al* in 1998(1) and is a validated surrogate marker of proliferation in lung cancer(1-4). In the cell, (1)FLT becomes monophosphorylated and trapped by the key thymidine salvage pathway enzyme, thymidine kinase 1 (TK1); thus tumors become more FLT-avid as thymidine salvage pathway activity increases. As such, this drug-induced salvage pathway "flare" effect provides an imaging opportunity to determine successful TS inhibition in the tumor. While prior studies have demonstrated that the "flare" can be imaged with FLT-PET *in vivo*,(5-9), these studies have been limited by alack of consensus on the exact timing of this effect and questions about its predictive value. For example, a TS inhibition-induced "flare" of the thymidine salvage pathway has been demonstrated at a range of time points from 1 to 48 hours using varying therapeutic combinations(5-11). Since this effect is transient and may vary significantly by drug type, characterization in each therapeutic setting is required prior to implementation in the clinic. Importantly, this technique has the potential to serve as a non-invasive biomarker of tumor response to TS-inhibition within hours of starting therapy.

The TS-inhibition induced FLT "flare" effect is thought to be mediated primarily though one or both of two mechanisms. The first is an increase in the activity(12, 13) and/or expression of TK1(7), the rate-limiting step of the thymidine salvage pathway. TK1 enzyme activity is modulated by its physical state, with its activity increasing with transition from monomer to dimer to tetramer state(14). The cellular

expression and activity of TK1 is carefully modulated throughout the cell cycle, peaking during S-phase, in order to maintain available dTTP pools for ongoing DNA synthesis(12, 15, 16). The inhibition of the *de novo* synthesis pathway resulting from TS inhibition appears to potentiate TK1 activity and expression in order to boost thymidine flux through the salvage pathway. Of note, studies proposing that changes in TK1 expression rather than activity are the primary mechanism of FLT "flare" have demonstrated a "flare" effect at least 24 - 72 hours following exposure to the TS inhibitor(17); in contrast, studies focusing on TK activity have noted changes within a few hours of drug exposure. These data suggest the possibility that differing mechanisms drive FLT accumulation in the cell depending on the timing of exposure to the drug and the specific types of therapeutics.

The other mechanism thought to contribute to the TS inhibition-mediated salvage pathway "flare" effect is changes in the equilibrative nucleoside transporter 1 (ENT1)(18) either from rapid mobilization to the cell surface or an increase in expression(13). Transport of thymidine through ENT1 is the primary mode of entry for thymidine for metabolism into the thymidine salvage pathway. Mobilization of ENT1 is regulated by the cell cycle(19), designed to increase transport of exogenous thymidine to meet the demands of the dividing cell. Although FLT, a thymidine analog, can enter the cell through passive diffusion or ENT1, ENT1 transport is a major mechanism of increased FLT entry for proliferating cells(19-21), and ENT1 is rapidly mobilized to the cell surface within hours of successful TS-inhibition(5, 18). However there is controversy regarding the role of ENT1 in the "flare" effect and several studies have failed to observe a shift in ENT1 to the cell surface following TS-inhibition(12). As with TK1, these conflicting findings may be related to differences in drug type or timing of the observed effect(12). Also, although ENT1 may contribute to the increased entry of FLT into the cell, ultimately intracellular retention of FLT is dependent on metabolism by TK1, the rate-limiting step of the thymidine salvage pathway(4, 15, 22).

To date the value of FLT "flare" imaging as a biomarker of TS inhibitor therapy response has been incompletely studied, especially with in the setting of pemetrexed therapy, a commonly used TS inhibitor in cancer and the 1st line chemotherapeutic for NSCLC (non-small cell lung cancer) and mesothelioma. Pemetrexed is an anti-folate that mediates inhibition of TS through its irreversible binding to the TS folate receptor. This is an effective strategy since reversible binding to both the dUMP (deoxyuridine monophosphate) and folate binding sites are necessary for TS function. By contrast, 5-FU, used for many of the studies of FLT "flare", inhibits TS through irreversible binding to the dUMP binding site(23). Several factors improve the efficiency of TS inhibition via pemetrexed compared to 5-FU including the fact that tumor cells are inherently folate-depleted, which increases access of pemetrexed metabolites to the TS binding site(24). Furthermore, successful inhibition of TS results in accumulation of dUMP(25, 26), which competes with 5-FU but not pemetrexed for TS binding sites.(24). Therefore the kinetics of the pemetrexed-induced salvage pathway "flare" likely differ from that of other TS-inhibitor agents such as 5-FU. Finally, although pemetrexed is a potent inhibitor of TS, it also inhibits the folate pathway enzyme dihydrofolate reductase (DHFR) and the *de novo* purine synthesis pathway enzyme glycinamide ribonucleotide transferase (GARFT)(24). This extra activity may impact the dynamics of pemetrexed-induced effects on thymidine metabolism. For these reasons, further characterization of kinetics of pemetrexed-induced thymidine salvage pathway "flare" is needed prior to translation to the clinic.

In this study, we define the kinetics of the pemetrexed-induced FLT "flare" in order to determine the optimal timing of FLT imaging for translation to the clinic. Furthermore, we elucidate the mechanism of FLT "flare" following pemetrexed-induced inhibition and characterize the potential impact of concurrent therapy with a platin drug on "flare" kinetics. This is important since pemetrexed regimens typically include a DNA-damaging platin agent such as carboplatin or cisplatin. Finally, we conduct a pilot trial of FLT-PET imaging of pemetrexed-induced TS inhibition in a patient with NSCLC to validate the feasibility this imaging technique at the determined optimal time point.

MATERIALS AND METHODS

Chemotherapeutics and imaging radiopharmaceuticals

For *in vitro* studies, pemetrexed (Santa Cruz Biotechnology, Dallas, Texas) and cisplatin (Sigma-Aldrich Corp., St. Louis, MO) were provided in solid form, dissolved in water and stored at -20^{o}C as a 0.2 mM and 1 mM stock, respectively. For *in vivo* use, both human and murine, pemetrexed (ALIMTA; Eli Lilly and Company, Indianapolis, IN) and cisplatin (Teva Pharmaceuticals, Petach Tikva Israel) were provided freshly prepared as a 1mg/ml sterile saline solution by the Abramson Cancer Center Pharmacy. For *in vivo* murine studies, chemotherapeutics were stored at 4^{o}C. [^{18}F]FLT was produced on site in the University of Pennsylvania PET Center Cyclotron facility. [^{18}F]FLT average specific activity was 5.32 +/- 2.14 Ci/umol, and radiochemical purity >99%.

Cell lines and culture

H460 and H1299 human non-small cell lung cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were grown in RPMI medium containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator in 5% CO2 at 37^{o}C. Passage of cell lines was performed at 1:3 dilution after detachment using sterile 0.05% trypsin-EDTA solution.

Mouse tumor xenograft modeling

Prior to *in vivo* animal modeling, approval was obtained by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Human tumor-bearing murine xenografts were then created using two month-old female *nu*/*nu* mice (Crl: NUFoxnlnu, Charles River Laboratory, Wilmington, MA). Each mouse was injected subcutaneously in the flank with a suspension of H460 cells (1×10^{7}) in sterile, endotoxin-free 50% Matrigel Matrix (Corning Inc., Corning, NY). When tumors

reached a mean volume of approximately 200 mm^3 (volume= π/6 × length × width × height), animals were randomized into treatment groups.

In vitro analysis of thymidine salvage pathway activity

^{3}H-thymidine assays

[^{3}H]-thymidine (Perkin Elmer NET355001MC, PerkinElmer, Waltham, MA) was utilized for *in vitro* assessment of therapy-induced changes in thymidine salvage pathway activity in cultured human NSCLC cells. [^{3}H]-thymidine specific activity was >10Ci(370GBq)/mmol and radiochemical purity >97%. H460 and H1299 cells were seeded (1×10^6/well) in 6-well plate in RPMI1640 supplemented with 10% FBS and antibiotics, incubated 24 hours in 5% CO_2 at 37^{o}C. When cell cultures achieved 80% confluence, cells were exposed to treatment with either the vehicle (sterile water), pemetrexed (100 nM), or the combination of pemetrexed (100 nM) and cisplatin (10 μM) in growth media for varying exposure times ranging up to 48hours. Drug-containing medium was then removed, and the cells were then washed and pulsed with 5 μCi [^{3}H]-thymidine/well for 1 h. The cells were then washed and scraped into plastic vials. Scintillant (10 ml; Research Products International Corp., Mount Prospect, IL) was added to each vial and the radioactivity was counted on a scintillation counter (Beckman Coulter LS6500, Beckman Coulter Life Sciences, Indianapolis, IN).

Immunoblotting

Therapy-induced changes in the protein expression of key components of the thymidine salvage pathway were assessed using Western blot analysis. H460 and H1299 cells were seeded (1×10^6 cells/well) in 6-well plate in RPMI1640 supplemented with 10% FBS and antibiotics, incubated 24 h in 5% CO_2 at 37℃. When cell cultures achieved 80% confluence, cells were exposed to treatment with either the vehicle (sterile water), pemetrexed (100 nM), or the combination of pemetrexed (100 nM)

and cisplatin (10 μM) in growth media for varying exposure times ranging up to 48 hours. Whole cell lysates were then generated using a 1% Nonidet P-40 lysis buffer (Sigma-Aldrich Corp., St. Louis, MO). The suspension was homogenized by passages through a 20-gauge syringe needle and nuclear material removed through centrifugation at 14000 rpm for 15 min at 4℃. Cell lysates were then loaded onto a precast Nupage Bis-Tris Gels (invitrogen, Life Technologies, Corp., Grand Island, NY) for electrophoresis then transferred onto Hybond-P PVDF membranes (Sigma-Aldrich Corp., St. Louis, MO) for Western blot analysis. After blocking membranes with 5% non-fat milk in PBS with 0.1% Tween-20 buffer, PVDF membranes were probed using primary antibodies directed against human TK1 (Cell Signaling Technology, Danvers, MA; 1:5000), human TS (Cell Signaling Technology; 1:4000), human ENT1 (Abcam, Cambridge, MA; 1:200), or human β-actin (Sigma-Aldrich Corp., St. Louis, MO; 1:10000). Membrane were then washed and incubated (1:3000) with species-specific secondary antibodies, either anti-rabbit or anti-mouse, conjugated to horseradish peroxidase (GE Healthcare Life Science; 1:3000) for 1 h, the proteins were detected using the Immobilon ECL system (EMD Millipore, Billerica, MA) and quantified using Image J software available through the National Institutes of Health (http://imagej.nih.gov/ij/).

Immunofluorescence

Therapy-induced changes in the intracellular localization of ENT1 were assessed using immunofluorescence. Cells were fixed with 4% paraformaldehyde at room temperature for 10 min. Cells were washed with PBS, permeabilized and blocked with 1% BSA / 10% normal goat serum / 0.3M glycine in 0.1% PBS-Tween solution for 1 h. Cells were then incubated with a primary antibodies directed to human ENT1 (Abcam, Cambridge, MA; 1:100) overnight at 4℃. After incubation with the primary antibody, cells were washed with PBS and incubated with Cy™2 AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA) with at room temperature for 1 h. DAPI (Thermo Fisher Scientific Inc., Grand Island, NY) was used to stain the cell

nuclei at a concentration of 0.5 μg/ml. For negative staining control, primary antibody was omitted during the procedure. Digital images were acquired using a Nikon E600 microscope. The results of the membrane positive immunofluorescence of ENT1 were semiquantitatively assessed and scored as predescribed(27). Scoring criteria were defined as follows: 0 for negative (0% to 5% staining); 1 for weakly positive (5% to 20% staining); 2 for moderately positive (20% to 50% staining); 3 and 4 for strongly positive (50% to 75% and ≥ 75%, respectively).

PET scanning and image analysis

Preclinical PET imaging

Baseline FLT-PET scans were performed on the day prior to therapy. Mice were then treated with either the vehicle control (i.p. sterile PBS) or combination therapy with pemetrexed (i.p. 100 mg/kg) and cisplatin (10 mg/kg). A post-therapy FLT-PET was then performed at varying time points following administration of therapy.

For the assessment tumor response to therapy, tumor-bearing animals were treated for a period of two weeks with 100 mg/kg pemetrexed (i.p. daily; days 1-5 and 8-12) and 10 mg/kg cisplatin (i.p. once weekly). During this treatment period, tumor volumes were estimated by external caliper measurements. After therapy/imaging completion, mice were euthanized with carbon dioxide inhalation.

After anesthesia with inhaled isofluorane in O_2 (3% induction, 1.5% maintenance), mice were injected intravenously with 300-350 mCi of [18F]FLT then allowed to recover from the anesthesia during the 60-min uptake time allowed for radiotracer accumulation. At 60 min post-injection, mice were anesthetized and imaged for a 15-min static acquisition on the small animal PET scanner (A-PET, built in collaboration with Philips Medical Systems) located at the University of Pennsylvania Small Animal Imaging Facility.

Human PET imaging

IRB approval was obtained through the University of Pennsylvania Institutional Review Board for use of FLT-PET/CT under an FDA IND in this clinical trial. An adult patient with unresectable non-small cell lung cancer (NSCLC) receiving therapy with pemetrexed and carboplatin was imaged with FLT-PET within 3 days prior to starting therapy and at 2 hours following the 1st administration of intravenous infusion of the chemotherapeutic regimen. Human FLT-PET/CT was performed on the Philips Ingenuity TF scanner (Philips Medical Systems, at the Perelman Center for Advanced Medicine at the University of Pennsylvania. FLT-PET static images were obtained after a 60 minute uptake time following intravenous injection of 5mCi of [18F]FLT.

Image analysis

For preclinical PET imaging, after acquisition, the images were reconstructed with the manufacturer software and tumor volumes of [^{18}F] FLT quantitated with the freely available Amide 3D software (LG Software Innovations), which allows for multiplanar analysis of the tumor volume. A region of interest (ROI) was traced around the tumor volume creating a 3D ROI that could be sculpted to the 3D tumor perimeter and visually inspected in the axial, coronal and sagittal planes. Statistical analysis of this 3D tumor uptake volume was then performed using Amide software including ROI volume (mm^3), mean counts/pixel, max PET counts/pixel and standard deviation of tumor counts/pixel. PET values generated by the A-PET machine are absolute pixel counts. In order to control for slight differences in radiotracer administration and *in vivo* biood distribution, absolute tumor counts were normalized to flank muscle (tumor ROI pixel value/thigh muscle ROI pixel value).

For human PET imaging, SUV_{MAX} of the primary NSCLC was measured from axial PET/CT fusion images at baseline and post-therapeutic FLT-PET/CT using Philips IntelliSpace Portal (v5.0.2.40009,

Philips Healthcare Nederland B.V., Netherlands) software. Post-therapeutic changes in tumor avidity for FLT-PET were then calculated.

Statistics

Analysis for statistical significance between 2 or more groups was performed using student T tests or ANOVA as appropriate. Kruskal-Wallis was performed to compare scoring of different time points for Immunofloresence of ENT1. P-values greater than 0.05 were considered significant. All analyses were performed with SPSS 19.0 (IBM Corp., Armonk, NY).

RESULTS

Pemetrexed-induced TS inhibition results in a "flare" in thymidine salvage pathway activity peaking at 2 hours *in vitro*

Initially, we sought to define the kinetics the TS inhibition-induced "flare" of the thymidine salvage pathway in NSCLC cells *in vitro*. Both H460 and H1299 NSCLC lines demonstrated a significant transient compensatory "burst" in thymidine salvage pathway activity peaking at 2 hours as measured on ^{3}H-thymidine assays (**Fig. 1**) relative to untreated controls. The magnitude of the thymidine salvage pathway "flare" at 2 hours was 39% over the baseline in H460 and 37% over baseline in H1299. The addition of cisplatin to pemetrexed treatment of these cells did not impact the amplitude or timing of the thymidine salvage pathway "flare" effect (H460: p=0.21; H1299: p=0.48). By 24 hours of therapy, both cells treated with pemetrexed and cells treated with a combination therapy of pemetrexed and cisplatin exhibited a significant suppression of proliferation relative to untreated controls. For cells treated with pemetrexed alone, intracellular ^{3}H-thymidine accumulation was decreased by 45% and 35% at 24 hours of exposure relative to baseline for H460 and H1299 respectively. At 24 hours of exposure to combination therapy with pemetrexed and cisplatin, intracellular ^{3}H-thymidine accumulation decreased by 81% and 60% relative to baseline for H460 and H1299 respectively. Significantly greater suppression of proliferation was exhibited by the combination therapy with cisplatin and pemetrexed versus pemetrexed alone (H460: p=0.002; H1299: p=0.011). Thus, for pemetrexed alone and in combination with cisplatin, the "flare" effect was optimal *in vitro* at 2 hours after starting therapy.

Pemetrexed-induced thymidine salvage pathway "flare" is a result of a combination of redistribution of ENT1 receptors and activation of TK1 to the tetramer state

In order to gain insights into the mechanism for the pemetrexed-induced thymidine salvage pathway "flare", the protein expression of several key components of the thymidine salvage pathway were examined following exposure to pemetrexed or pemetrexed plus cisplatin. TK1, rate-limiting enzyme of the thymidine salvage pathway, exists in a modestly active monomer, moderately active dimer and highly active tetramer state. Treatment of both H460 and H1299 cells revealed a shift in TK1 protein to the tetramer state following 2 hours of exposure to pemetrexed therapy, with an increase of TK1 in the tetramer state of 10 fold ($p < 0.0001$) and 1.6 fold ($p=0.011$), respectively, and corresponding decreases in the monomer and dimer states of TK1 **(Fig. 2)**. These data are consistent with the timing of the pemetrexed-induced "flare" in thymidine salvage pathway activity observed at 2 hours using ^{3}H-thymidine assays (**Fig. 1**). Similar to data from the ^{3}H-thymidine assay data, the induction of this tetramer state was unaffected by exposure to cisplatin (H460: $p=0.48$; H1299: $p=0.70$).

Levels of total TS and ENT1 protein expression were not significantly changed during the 1st 8 hours of treatment. However, by 16-24 hours of exposure of cells to pemetrexed plus cisplatin, both cell lines exhibited a gradual decrease in protein expression of TK1, TS and ENT1 in keeping with successful cell cycle arrest. At 24 hours, there was widespread decrease in expression in the measured proteins relative to baseline. TK1 (total protein) was decreased by 36% ($p=0.0092$) and 35% ($p=0.007$), TS decreased by 38% ($p=0.04$) and 8.67% ($p=0.64$), and ENT1 decreased by 17% ($p=0.19$) and 84% ($p=0.0005$) for H460 and H1299 respectively.

In addition to examination of protein expression of key elements of the thymidine salvage pathway, we also examined subcellular localization of ENT1. By Immunofloresence microscopy, we detected a transient but significant shift in subcellular localization of ENT1 protein from the peri-nuclear location to the cell surface following treatment with pemetrexed. Cell-surface localization of ENT1 peaked at 2 hours of exposure to pemetrexed with a 3.9 fold ($p<0.0001$) and 4.2 fold ($p<0.0001$) increase for

H460 and H1299 respectively. This peak was followed by a steady shift back to peri-nuclear localization with a return to near baseline distribution by 24 hours (**Fig. 3**).

***In vivo* modeling validates *in vitro* kinetics of pemetrexed-induced "flare" in thymidine salvage pathway activity**

Prior to conducting a study of FLT flare kinetics, we confirmed efficacy of combined therapy with pemetrexed and cisplatin *in vivo*. A pilot study of 12 H460 tumor-bearing xenografts, 4 control (vehicle only) and 8 treated (combination of pemetrexed and cisplatin), revealed tumor growth inhibition of 88% in those treated with pemetrexed and cisplatin relative to vehicle only controls (*data not shown*). Consequently, *in vivo* modeling of 1st line therapy of NSCLC xenografts with combined pemetrexed and cisplatin therapy revealed a peak in tumor avidity for FLT at 2 hours of exposure to therapy (**Fig. 4**). At 2 hours of therapy, tumor FLT_{MAX} increased 47.5% (±12.0%, p=0.008) relative to baseline in the xenografts treated with cisplatin and pemetrexed but remained unchanged in vehicle treated controls (p=0.37). After this post-therapeutic peak or "flare" in tumor avidity for FLT at 2 hours, tumor avidity for FLT decreased over the course of 24 hours. By 24 hours following start of combination therapy with pemetrexed plus cisplatin, tumor avidity for FLT began to fall below baseline, albeit not significantly, with a mean tumor FLT_{MAX} of -6.0% (±10.5%, p=0.35). This is likely due to the impact of successful therapy on proliferation, which occurs in a similar timeframe to the *in vitro* studies of thymidine salvage pathway activity and protein expression **(Fig. 1 and 2).**

Human FLT-PET imaging of pemetrexed-induced "flare" at 2 hours of therapy is feasible

We next sought to extend our findings to human lung cancer through a proof-of-concept clinical trial of FLT-PET/CT as a measure of TS inhibition in patients with advanced NSCLC. Baseline FLT-PET performed three days prior to start of therapy revealed a FLT-avid (SUV_{MAX} of 5.4) solid 6 cm mass in the left upper lobe in keeping with the known NSCLC. A repeat FLT-PET/CT scan performed at 2

hours following the first infusion of combined therapy with pemetrexed and carboplatin revealed a post-therapeutic increase in tumor avidity for FLT with a 44% increase in SUVmax (SUV_{MAX} of 7.8) relative to baseline (**Fig. 5**). Thus, in a human patient with NSCLC, FLT-PET revealed a "flare" in tumor avidity for FLT at 2 hours following intravenous infusion with pemetrexed and carboplatin; this study demonstrates the feasibility of translating this technique, using a 2 hour time point to the clinic. Nonetheless, more patients will need to be studied to validate the predictive value of this post-therapeutic FLT "flare" for lung cancer patients treated with 1st line pemetrexed-based therapies.

DISCUSSION

In this study we have determined the optimal timing for imaging the pemetrexed-induced thymidine salvage pathway "flare" in NSCLC. This is important since most imaging of the TS-inhibition induced thymidine salvage pathway "flare" has been performed using TS inhibitors other than pemetrexed. *In vitro* and *in vivo* data in this study consistently show a transient peak in thymidine salvage pathway "flare" activity at 2 hours of exposure to pemetrexed. This timing of the thymidine salvage pathway "flare" is in the range of that reported in the literature for other TS inhibitors but earlier than the time points used in other clinical trials of TS inhibitor induced FLT "flare" (9, 10). By optimizing the timing of the FLT "flare" in pemetrexed therapy, future clinical trials will be positioned to study the predictive value of this imaging strategy in this therapeutic setting. Moreover, to our knowledge, this is the first study to determine that concurrent treatment with cisplatin does not alter the timing or magnitude of the TS-inhibition mediated salvage pathway "flare." This is critical to clinical translation of this imaging strategy since pemetrexed is frequently used in combination with a platin-based therapeutic for oncologic management.

Tumor avidity for FLT is well correlated with expression(28) and activity(15) of the rate-limiting step in the thymidine salvage pathway, TK1(28), and with the cell surface expression of nucleoside transporter, ENT1(19, 20). Analysis of protein expression in this study suggests that the TS inhibitor-induced thymidine salvage "flare" effect is likely attributable, at least partially, to an increase in TK1 activity via a shift in enzyme state to highly active tetramer. It is less likely that the salvage pathway "flare" effect is mediated by changes in TK1 or ENT1 protein expression since changes in protein expression are unlikely to occur in a 2 hour time interval. Lee et al described a bimodal FLT "flare" occurring at 2 hours and 24 hours in most cell lines treated with 5-FU(7). This later 24-48 hour peak in salvage pathway "flare" may be due to increased expression of TK1(5, 7) or ENT1(13), which is observed following monotherapy with 5-FU(7, 13) or BGC 945(5). We hypothesize that the nature of

the therapeutics, such as the choice of TS inhibitor and use of monotherapy versus combination therapy, may impact contributions of available salvage pathway compensatory mechanisms resulting in differing FLT “flare” kinetics. As such, we posit that our use of concurrent therapy with a DNA damaging platin agent likely prevents compensation of the DNA salvage pathway through increases in protein expression, seen in some models at 24-48 hours, due to overall suppression of protein expression.

Our data also indicate that transient localization of ENT1 to the cell surface likely contributes to the thymidine salvage pathway “flare” effect. There is support in the literature for both increases in TK1 activity(7, 12, 13) and redistribution of ENT1 to the cell surface(5, 18) as potential mechanisms for the TS-inhibition mediated thymidine salvage pathway “flare” effect. The ENT1 transporters may also contribute to the subsequent decrease in tumor avidity for FLT following the “flare” as they have been demonstrated to contribute to nucleoside/nucleotide efflux from the cell(29) and to be decreased in expression as cells exit active cell cycling(19) in response to therapy. In addition, other nucleoside equilibrative and concentrative transporters are known to contribute to FLT metabolism(20, 29) and may also play a role in the dynamics of pemetrexed-induced FLT “flare”.

Our study results support previously published data that the TS-inhibition induced “flare” in thymidine salvage pathway activity rapidly dissipates. Using pemetrexed therapy, the “flare” effect was gone by 24 hours both *in vitro* and *in vivo* likely because, by 24 hours, the effects of the chemotherapeutics have begun to impair tumor proliferation with resulting suppression of thymidine salvage pathway activity and protein synthesis. These findings are compatible with the findings of Barthel *et al* who demonstrated that tumor avidity for FLT is significantly decreased relative to baseline by 48 hours of exposure to 5-FU(30). This anti-proliferative effect is greater with combined therapy of pemetrexed and cisplatin compared to pemetrexed alone, as would be expected in these cell lines that are sensitive to both pemetrexed and cisplatin. In light of these data we propose that an imaging strategy

designed to specifically detect the success of pemetrexed-induced TS inhibition should measure thymidine salvage pathway at 2 hours. In contrast, imaging designed to evaluate the impact of pemetrexed-induced effects on proliferation should measure thymidine salvage pathway activity after 24 hours of therapy. Consequently, we anticipated that our data will inform clinical trials of imaging of TS inhibition using imaging markers of thymidine salvage pathway activity such as FLT.

Finally, our pilot clinical trial of the FLT “flare” imaging strategy in a patient with NSCLC demonstrates that imaging at the 2 hour window following infusion with pemetrexed-based therapy is feasible in the clinic and a clear “flare” is easily visualized. In sum, our data provide a rationale for using the 2 hour “flare” time point as the foundation for a clinical trial of FLT-PET as an imaging biomarker of response to 1st-line therapy in NSCLC and mesothelioma, which could dramatically alter management of these cancers.

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FIGURE LEGENDS

Figure 1. Pemetrexed-induced TS inhibition results in a "flare" of the thymidine salvage pathway activity. ^{3}H-thymidine assay was performed on PEM-sensitive NSCLC H460 in untreated control (culture medium only), pemetrexed (100nM) and combination therapy with pemetrexed (100nM) plus cisplatin (10mM). A "flare" of thymidine salvage pathway activity peaked at 2 hours of pemetrexed therapy exposure.

Figure 2. Induction of tetramer state of TK1 corresponds temporally with pemetrexed-induced thymidine salvage pathway "flare." A time course of exposure of PEM-sensitive NSCLC cell line H460 to combination therapy with PEM/cisplatin *in vitro* revealed induction of highly activated tetramer TK1 state corresponding temporally to the FLT "flare" observed at 2 hours. ENT1 and TS protein levels remain unchanged in the "flare" period. All protein levels slowly decreased beyond 8 hours due to successful cell cycle inhibition by PEM/cisplatin therapy.

Figure 3. Translocation of ENT1 to the cell surface corresponds temporally with the pemetrexed-induced thymidine salvage pathway "flare." Immunofloresence microscopy utilizing a time course of exposure of the pemetrexed (PEM)-sensitive NSCLC cell line H460 to combination therapy with PEM(100nM)/cisplatin(10 μM) in vitro revealed translocation of ENT1 to the cell surface from the peri-nuclear cytoplasm maximal at 2 hours of exposure to PEM or PEM/cisplatin corresponding to the timing of the FLT "flare". (a.) cells were scored on a scale 0 (no ENT1 translocation) to 5 (high ENT1 translocation to the cell surface) Microscopy demonstrated NSCLC staining for ENT1 (Green stain) and nuclear membrane (blue stain) following 2 hours of exposure to (b.) culture medium control (c.) or combination of pemetrexed and cisplatin. Arrows depict concentration of ENT1 staining

Figure 4. FLT-PET imaging of pemetrexed-induced TS inhibition demonstrates a FLT "flare" peaking at 2 hours in a preclinical model of NSCLC. Human NSCLC tumor-bearing murine xenografts were treated with a combination of pemetrexed (PEM) and cisplatin in order to model 1st line therapy. FLT-PET was performed at baseline and at multiple time points following therapy start. Tumor avidity for FLT was observed to peak at 2 hours following PEM-based therapy. By 24 hours of therapy, tumors began to demonstrate inhibition of proliferation. (b) excreted radiotracer within the bladder.

Figure 5. FLT "flare" in response to PEM-based therapy in a human patient. These images are from a 63 year-old male with NSCLC participating in our exploratory clinical trial of FLT-PET "flare". (a) Baseline CT revealed a 6 cm mass in the left upper lobe. (b) Baseline FLT-PET revealed mild avidity 3 days pre-therapy. (c) FLT-PET "flare" imaging performed after 2 hours following administration of combination therapy with PEM and carboplatin revealed a burst in tumor avidity in keeping with the FLT "flare."

FIGURES

Figure 1.

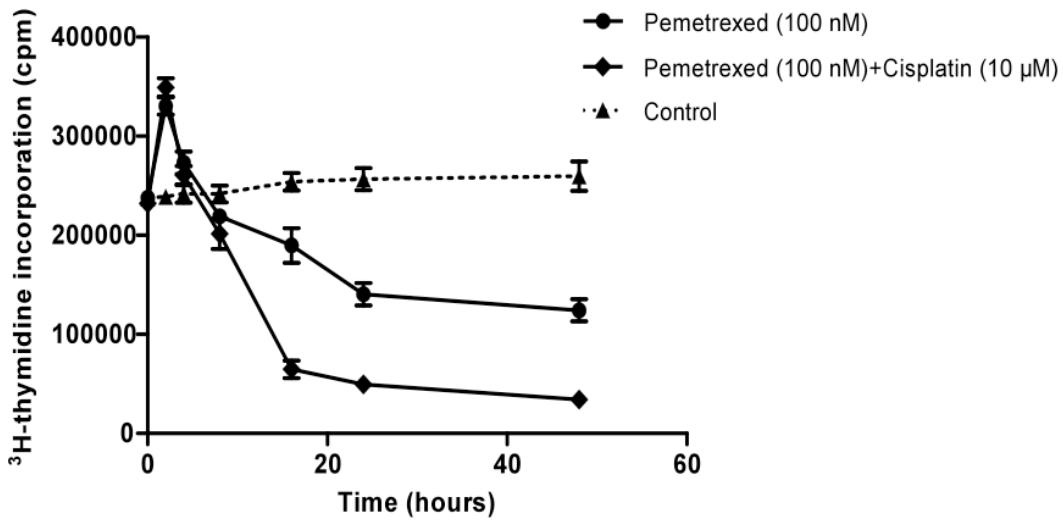


Figure 2.

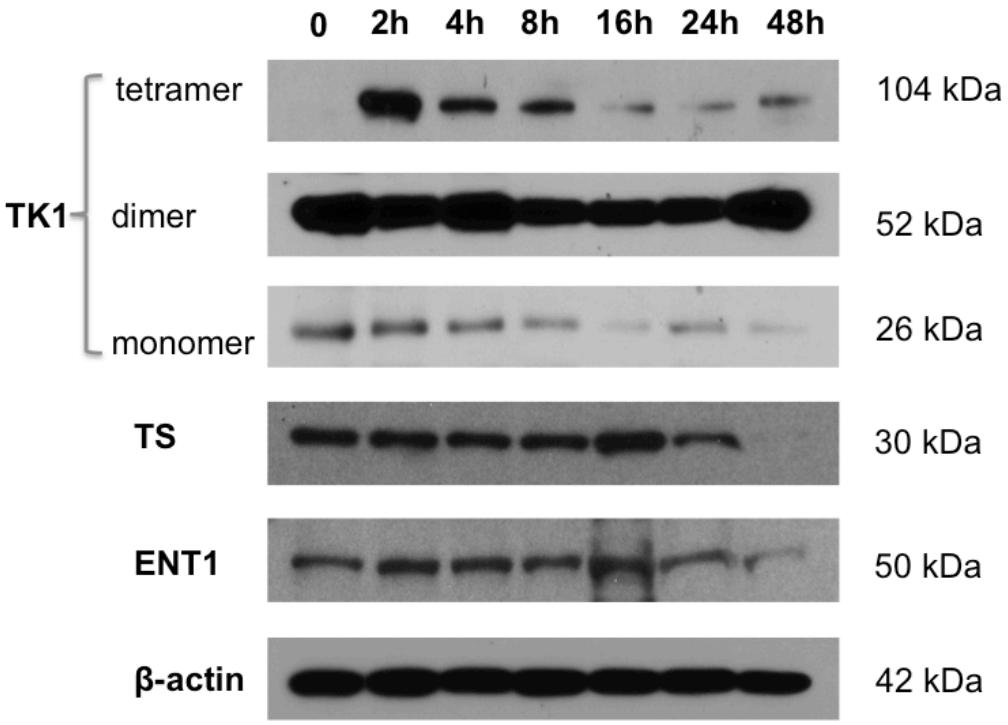


Figure 3.

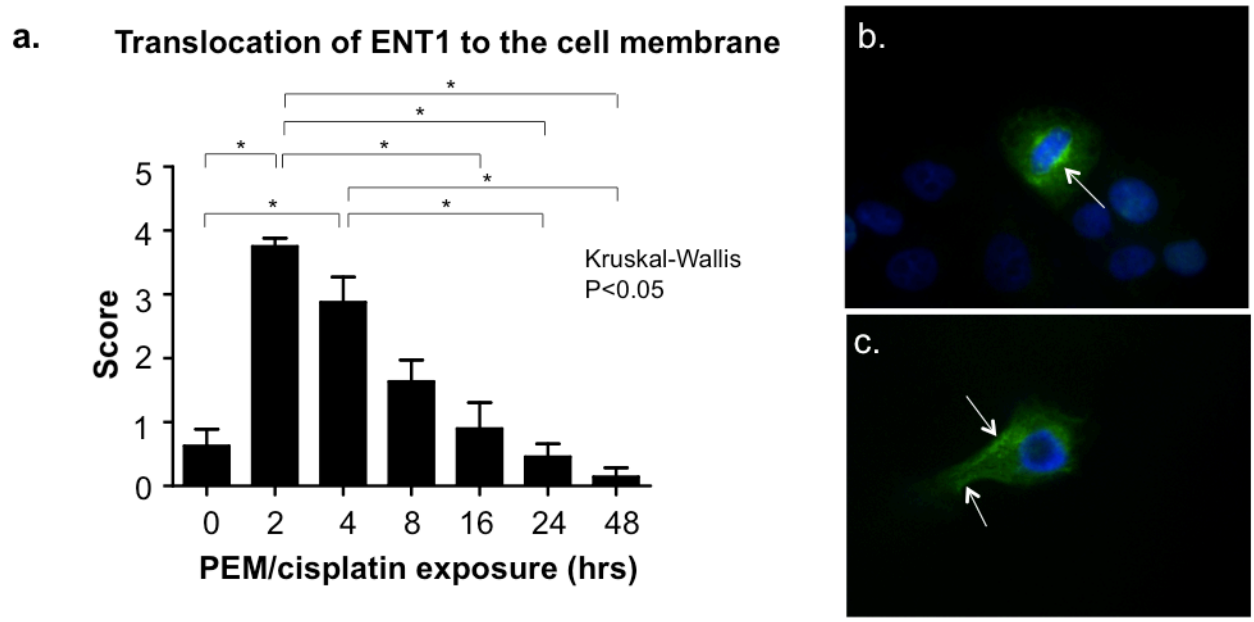


Figure 4

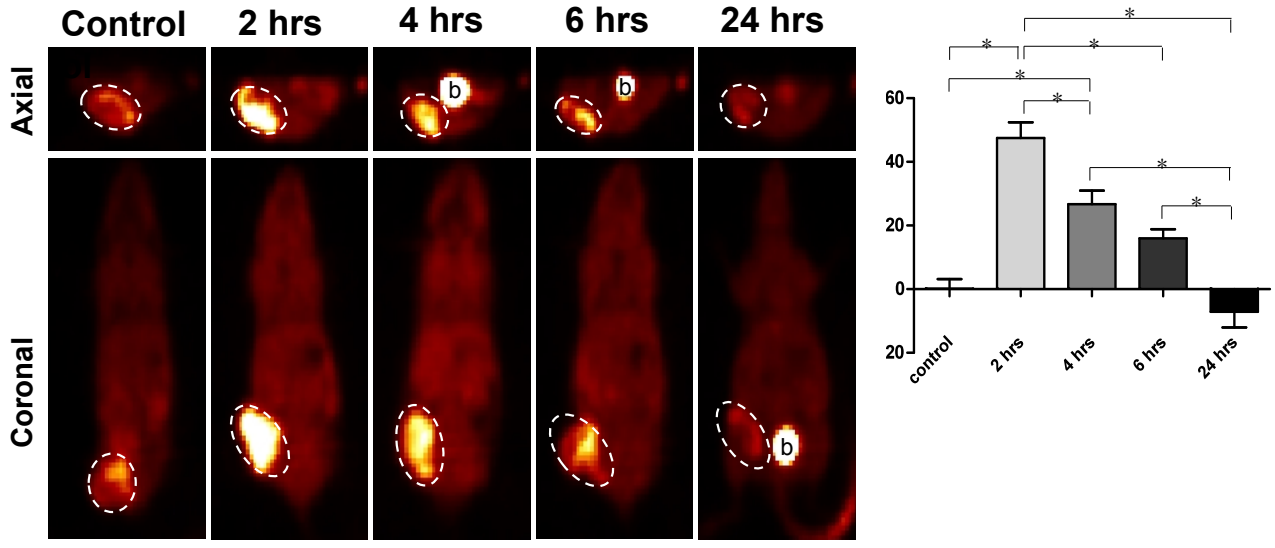
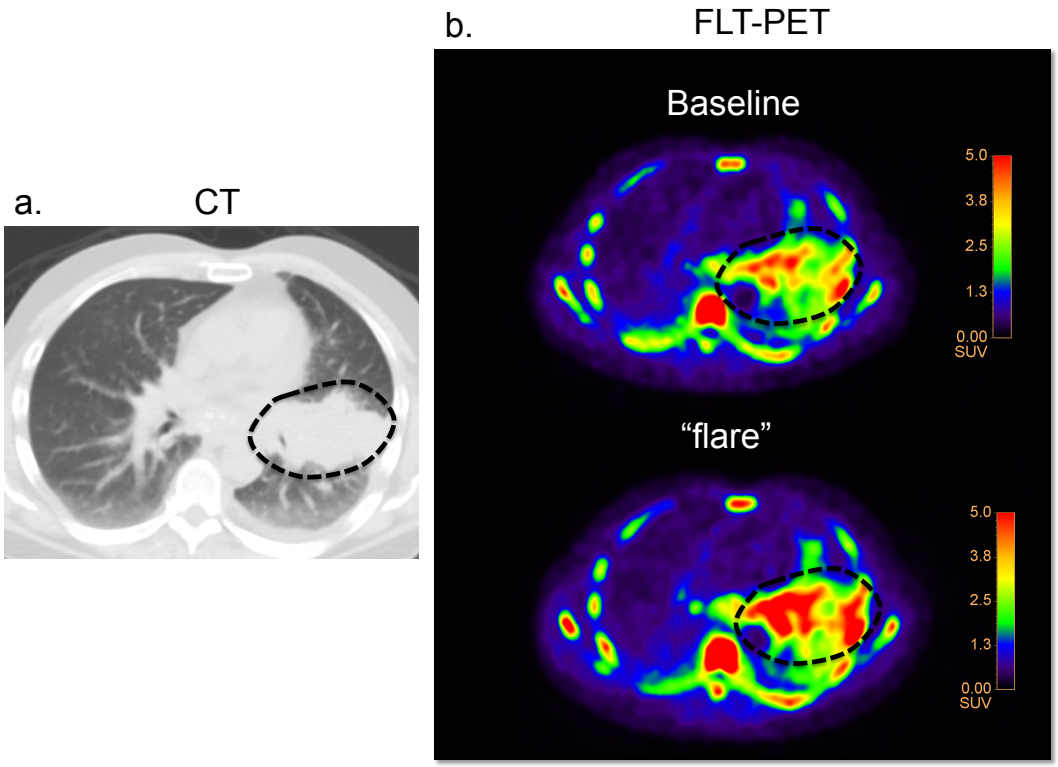


Figure 5.


a.
CT
b.
FLT-PET
Baseline
5.0
3.8
2.5
1.3
0.00
SUV
“flare”
5.0
3.8
2.5
1.3
0.00
SUV

Appendix 2: Abstract for the AACR annual meeting 2016

Title: Early Detection of Pemetrexed-induced Inhibition of Thymidylate Synthase in Non-Small Cell Lung Cancer with FLT-PET imaging.

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Purpose: Inhibition of thymidylate synthase (TS) results in a transient burst or "flare" in DNA thymidine salvage pathway activity measurable with FLT ([^{18}F]thymidine)-positron emission tomography (PET), allowing a measure of tumor response to TS inhibition on day 1 of therapy. Here we characterize this imaging strategy for implementation into 1st line pemetrexed-based therapy for non-small cell lung cancer (NSCLC) or mesothelioma.

Experimental Design: Since pemetrexed acts by inhibiting TS, we defined the kinetics of increases in thymidine salvage pathway mediated by TS inhibition following treatment with pemetrexed *in vitro*. Next, using a mouse model of NSCLC, we validated the kinetics of the pemetrexed-mediated "flare" in thymidine salvage pathway activity *in vivo* using FLT-PET imaging. Finally, we translated our findings into a proof-of-principle clinical trial of FLT-PET in a human NSCLC patient.

Results: In NSCLC cells *in vitro*, we identified a burst in thymidine salvage pathway activity, assessed by ^{3}H-thymidine assays, thymidine kinase 1 (TK1) expression, and equilibrative nucleoside transporter 1 (ENT1) mobilization to the cell membrane, that peaked two hours after pemetrexed treatment. Addition of cisplatin did not impact the amplitude or timing of this pemetrexed-induced "flare". This two-hour time-point was also optimal for FLT-PET imaging of pemetrexed-mediated TS inhibition in murine xenograft tumors. Imaging of pemetrexed-induced TS inhibition in a NSCLC patient demonstrated feasibility at the 2 hour time point.

Conclusions: FLT-PET measured efficacy of pemetrexed-induced TS inhibition is optimal at 2 hours from the start of therapy; this timing is feasible in human clinical trials.